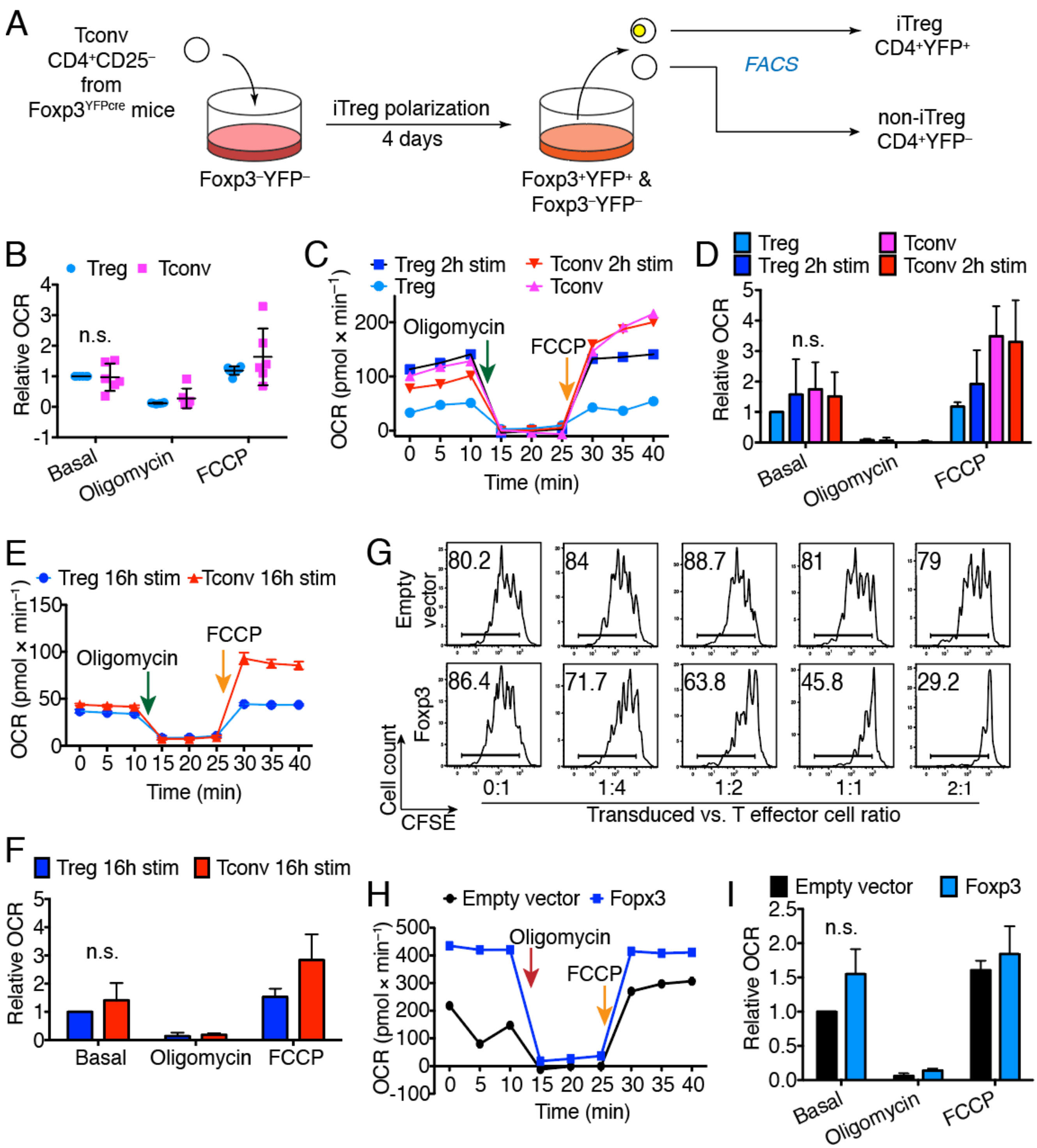
Supplemental Information

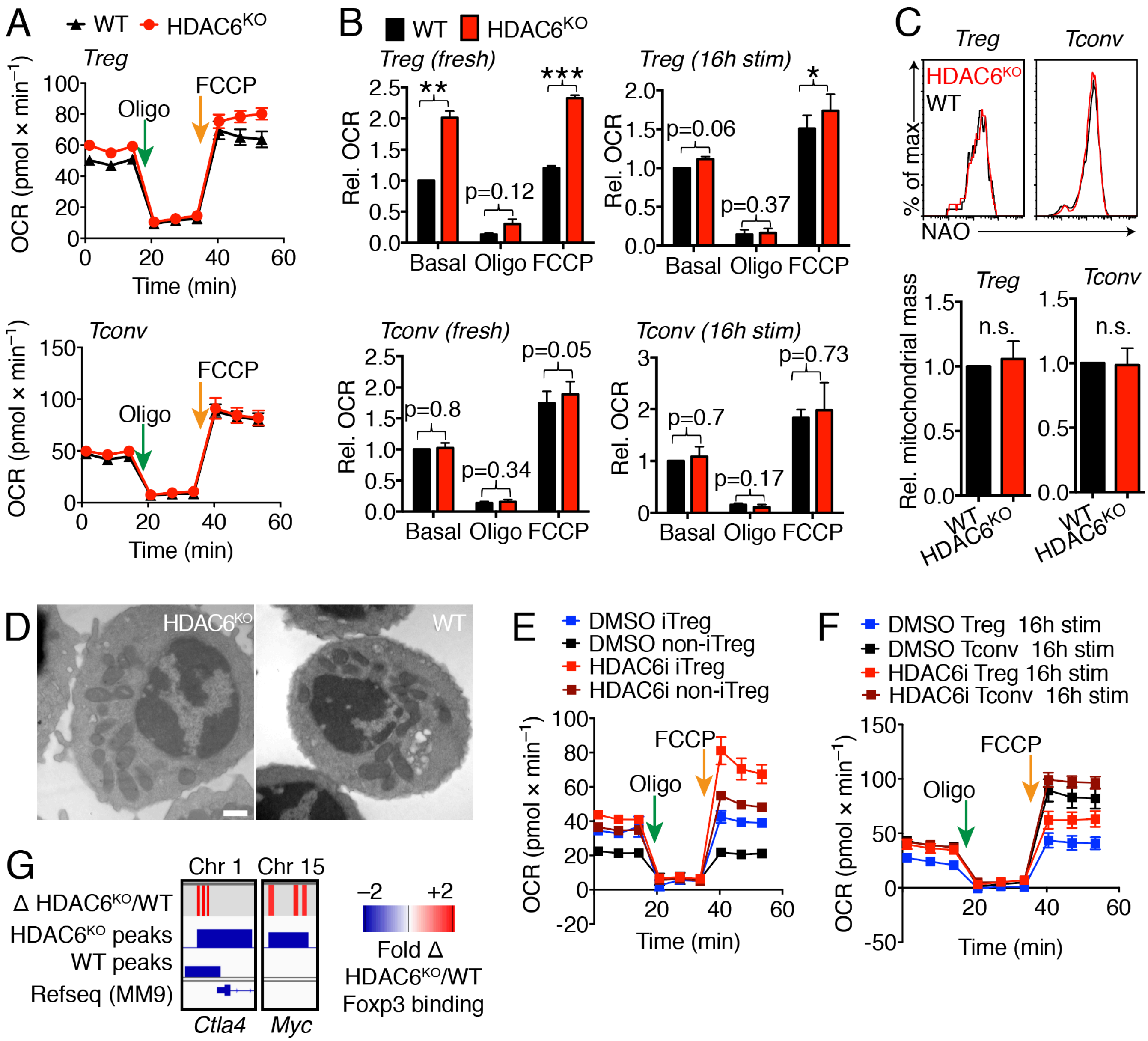
Supplemental Data Items

Figure S1: Oxygen consumption in naïve and stimulated Tconv and Treg. Related to Figure 1.



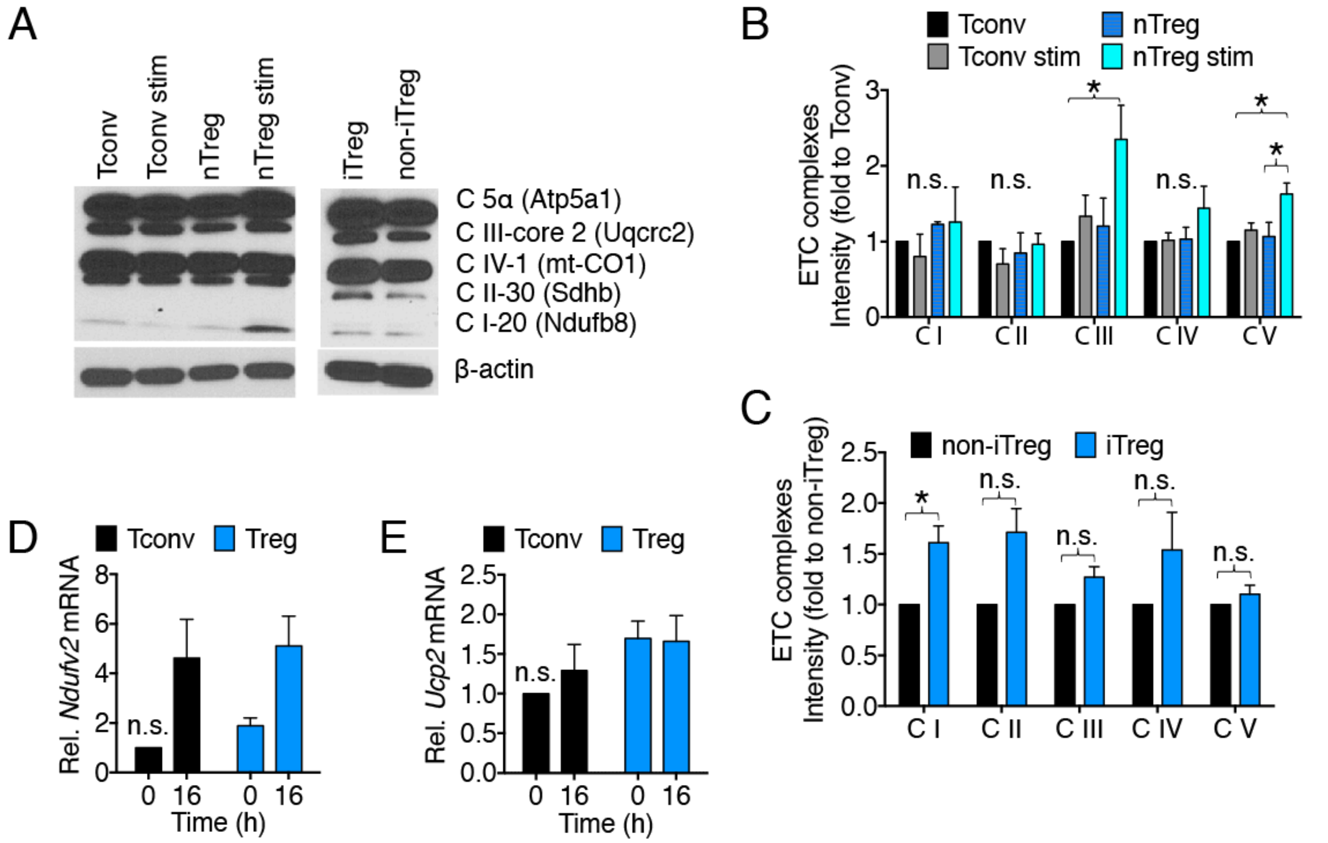
(A) $CD4^+CD25^-$ Tconv from $Foxp3^{YFPcre}$ mice were stimulated and polarized to form iTreg. T cells that acquired Foxp3 were identified using yellow fluorescent protein (YFP), and isolated using fluorescence-activated cell sorting (FACS). At every step, an aliquot of T cells was stained for Foxp3 purity (which requires fixation and permeabilization) to assess the proportions of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T converted and isolated cells (see Fig. 1A). The FACS purified 'iTreg' and 'non-iTreg' cells were subjected to seahorse bioenergetics measurements and other studies. (B) Bioenergetics profiles using seahorse mitochondrial function testing comparing freshly isolated C57BL/6 $CD4^+CD25^+$ Treg to Tconv. Data pooled from six independent experiments and normalized to the basal Treg respiration of each pair. No significant differences were observed (paired Student's t-test). (C & D): C57BL/6 Tconv and Treg were co-stimulated for 2 h with platebound CD3ε/CD28 mAb, or kept on ice for 2 h. (C) Representative and (D) cumulative seahorse mitochondrial function testing showed a trend to higher OCR in stimulated Tconv and Treg, although not significant (n.s.). Data pooled from three independent experiments (paired Student t-test). (E & F): Tconv and Treg were isolated from C57BL/6 mice and co-stimulated for 16 h with CD3ε/CD28 mAb coated beads, as well as 25 U × mL^{-1} IL-2. (E) Representative and (F) cumulative data showed that stimulated Tconv showed a trend to higher OCR relative to stimulated Treg (paired Student t-test). Data pooled from three independent experiments. (G-I) C57BL/6 $CD4^+$ T cells were transduced with MinR1-Foxp3 or empty vector constructs, as previous described (Liu et al., 2012). The transduced T cells were used as suppressive cells in a Treg suppression assay (as described in Fig 3C). (G) Foxp3, but not empty vector-transduced T cells acquired a suppressive phenotype resembling regulatory T cells. (H) Seahorse mitochondrial function testing showed that Foxp3 transduced T cells show increased OCR. (I): Pooled seahorse data from five independent experiments shows a trend towards increased OCR after Foxp3 transduction (paired Student t-test). Error bars indicate SEM.

Figure S2: HDAC6 deletion and inhibition increases T cell oxygen consumption. Related to Figure 1 and 2.



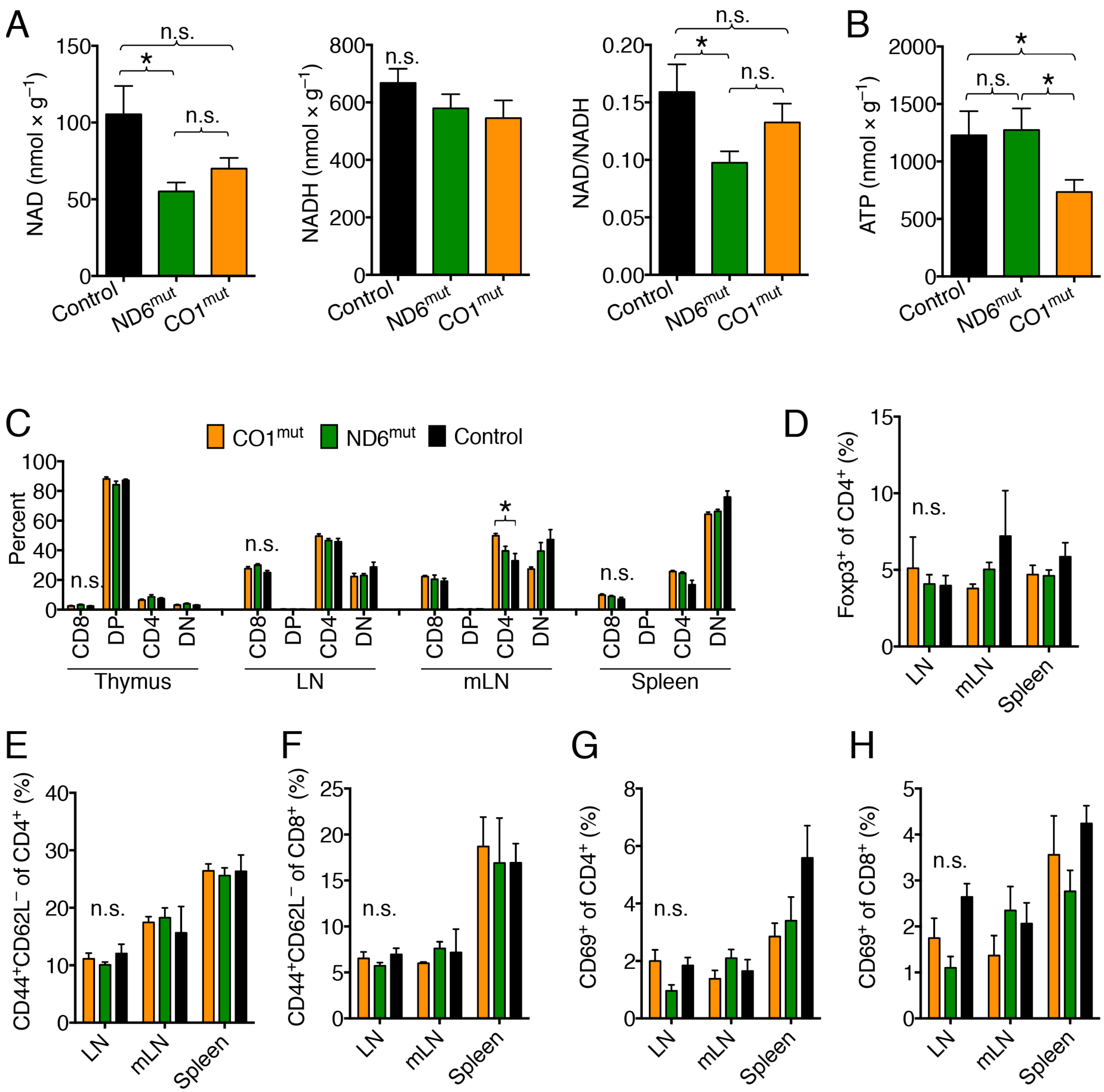
(A, B) Treg (CD4$^+$CD25$^+$) and Tconv (CD4$^+$CD25$^-$) were isolated from C57BL/6 splenocytes from mice with or without HDAC6 deletion (wild type, WT and HDAC6^{KO}) and tested for mitochondrial function analogous to Figure 1B. HDAC6^{KO} Treg, but not Tconv, showed increased OCR (B, left panels, 4/group). After 16 h co-stimulation with CD3/CD28 mAb coated beads, Treg still showed a small increase in OCR, yet the difference was much reduced (B, right panels, 3/group). *, ** and *** indicate $p<0.05$, $p<0.01$ and $P<0.001$, respectively (paired Student t-test). (C) Nonyl acridine orange (NAO) staining by flow cytometry shows equal mitochondrial mass between HDAC6^{KO} and wild type controls. Data pooled from six independent experiments. (D) Transmission electron microscopic image of purified HDAC6^{KO} and WT Tregs shows normal mitochondria without overt pathology and equal mitochondria count per cell. Scale bar indicates 500 nanometers. (E) CD4^{+}CD25^{-} Tconv from Foxp3^{YFPcre} mice were stimulated and polarized to form iTreg as outlined in Fig 1A, B and S1A. During iTreg polarization, we added the HDAC6 inhibitor (HDAC6i) tubastatin (Butler et al., 2010) at 0.5 μM, versus DMSO loading control. 0.5 μM tubastatin has been previously shown to augment Treg function and stability (de Zoeten et al., 2011). Induced Treg, as well as the non-iTreg control both exhibited an increase in OCR. Likewise, DMSO treated iTerg showed higher OCR compared to the non-iTreg control, consistent with Fig 1B, C. Data representative of three independent experiments. (F) Likewise, DMSO and HDAC6i treatment of 16 h stimulated Tconv and Treg (see supplemental figure S1E, F) also showed an increase in OCR in both Tconv and Treg from HDAC6 inhibitor treatment. Data representative of two experiments. (G) ChIP seq assay of HDAC6^{KO} and WT CD4^{+}CD25^{+} Treg pulling down with Foxp3 mAb identified multiple potential Foxp3 bindings to the Myc promoter. Data pooled from triplicate repeat and visualized with the Broad Institutes Integrative Genomics Viewer. Error bars indicate SEM.

Figure S3: Electron transport chain complexs in Tconv and Treg. Related to Figure 2.



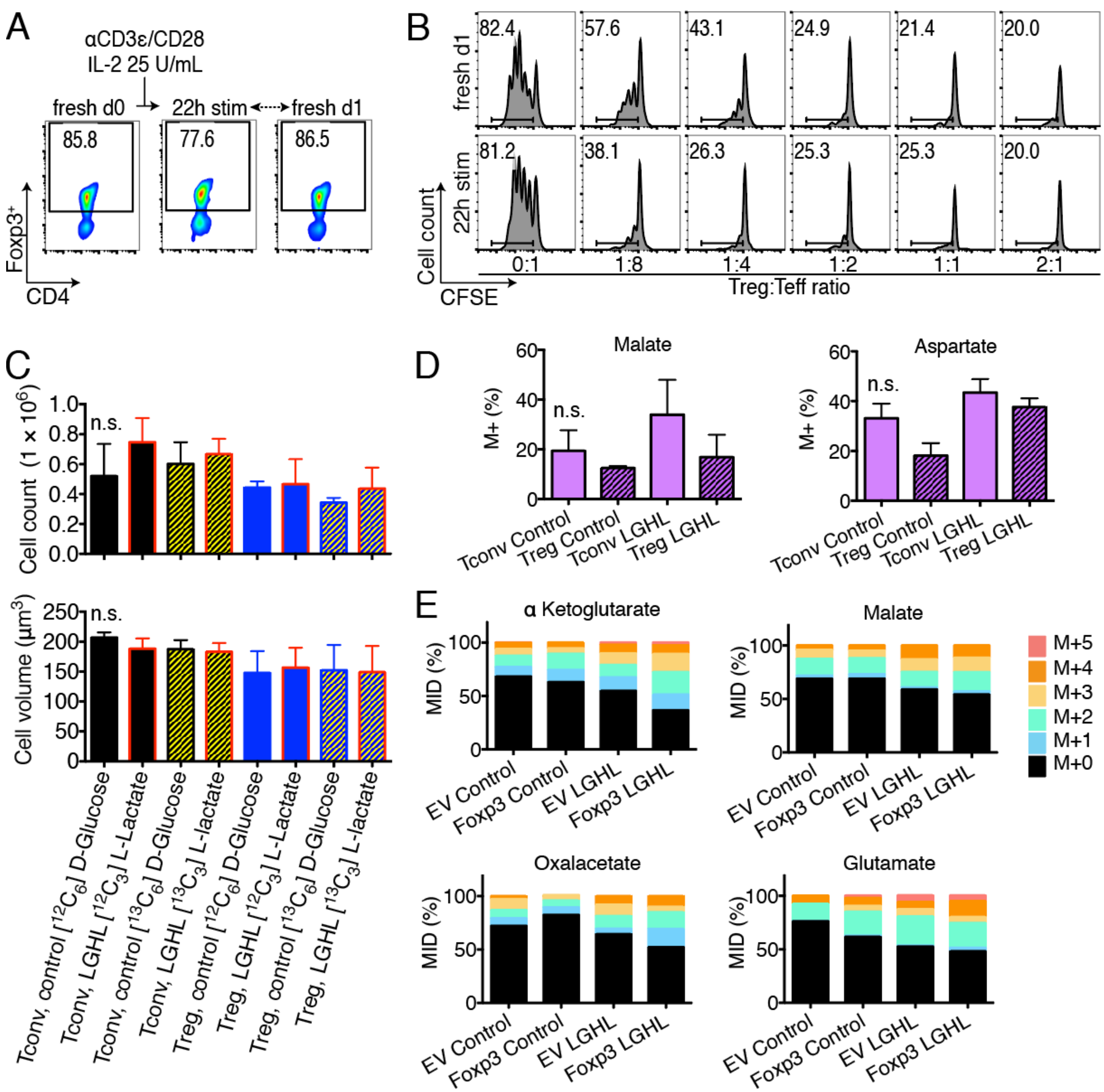
(A-C) $CD4^{+}CD25^{-}$ Tconv were stimulated with CD3ε/CD28 mAb coated beads and 25 U × mL^{-1} IL-2 for 16 h. $CD4^{+}CD25^{-}$ Tconv from $Foxp3^{YFPcre}$ mice were stimulated and polarized to form iTreg and then separated from non-iTreg using fluorescence-activated cell sorting (see supplemental figure S1A). Representative (A) and cumulative (4-6/group) (B, C) Western blotting shows that iTreg exhibit more electron transport chain (ETC) complex I expression than non-iTreg (C), which was not seen in 16 h activated thymic/natural Treg (nTreg). (D, E) Quantitative PCR of *Ndufv2* (D) and *Ucp2* (E) mRNA in freshly isolated and 16 h activated Tconv and Treg (3/group). * indicates $p<0.05$ (paired Student t-test), n.s. not significant. Error bars indicate SEM.

Figure S4: Loss of mitochondrial ND6 and CO1 leads to reduced NAD oxidation and ATP production, respectively, without defects in T cell development. Related to Figure 3.



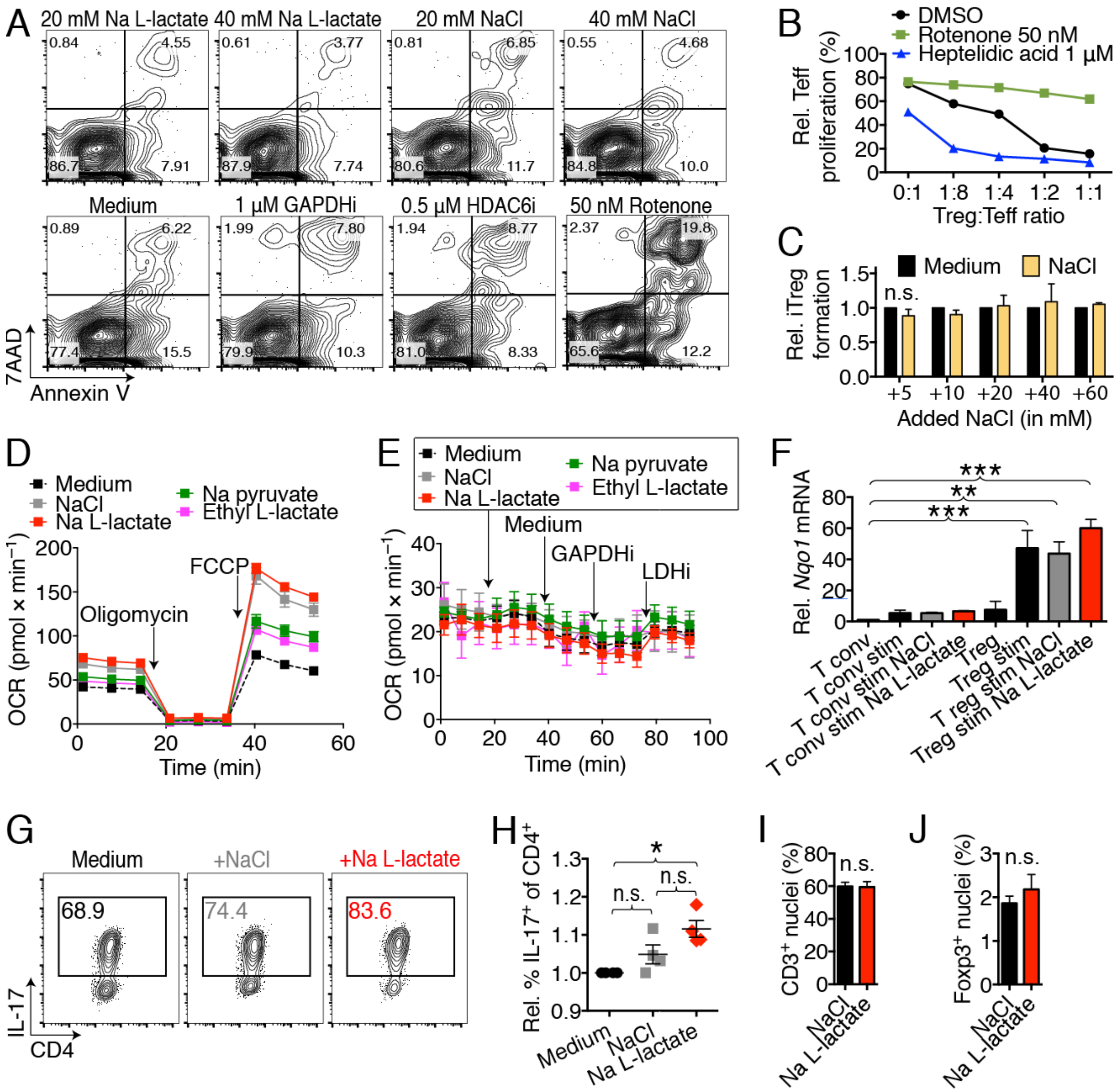
(A, B) Liver NAD and NADH (A), as well as ATP (B) production shows that loss of ND6 (ETC complex I) and CO1 (ETC complex IV) cause reduced NADH to NAD oxidation in ND6^{mut}, and diminished ATP production in CO1^{mut} mice, respectively. (C-H): Baseline T cell phenotyping. Lymphatic tissues from ND6^{mut} and CO1^{mut} mice show equal CD4 and CD8 T cell populations (C), equal CD4^{+}Foxp3^{+} Treg (D), as well as comparable subsets of naïve and effector memory T cell populations (E-H). Data generated from 4-6 mice per group, significance indicated as unpaired one-way ANOVA, with * indicating $p<0.05$. Error bars indicate SEM. Abbreviations: DP, double positive (CD4^{+}CD8^{+}); DN, double negative (CD4^{-}CD8^{-}); LN, somatic lymphnodes; mLN, mesenteric lymphnodes; n.s., not significant.

Figure S5: Tricaboxylic acid derivatives in activated effector and regulatory T cells. Related to Figure 4.



(A) $CD4^+CD25^+$ Treg were isolated with 85.8% $Foxp3^+$ purity, and co-stimulated overnight with CD3/CD28 mAb coated beads and 25 U × mL^{-1} IL-2. On the next morning, another set of Tregs (and Tconv and antigen presenting cells) was isolated, with comparable $Foxp3^+$ purity (86.5%). At the end of isolation of the new Treg, a sample of the by now 22 hour stimulated Treg was tested for $Foxp3^+$ retention. Despite a minor reduction in $Foxp3^+$ expression (77.6%), the 22 hour stimulated Treg retained strong suppressive function (B). (C) Cell count and cell volume measurements of stimulated Tconv and Treg used for LC/MS experiments in Figure 5 after stimulation and labeling. Starting cell count was 1×10^6 for all conditions. Data derived from three independent experiments, paired one-way ANOVA. (D) Additional tricarboxylic acid derivatives from the ultra-high-performance liquid chromatography-selected reaction monitoring/mass spectrometry experiments reported in Figure 5. M+ (%) indicates the percentage of molecules that have one or more [^{13}C] after background correction. Data derived from three independent experiments (paired Student t-test). (E) Foxp3 and empty vector transduced T cells (see supplemental figure S1G-I, Figure 4A) were subjected to the same experimental conditions as Tregs and Tconv in Figure 5, respectively. M+ indicates the number of [^{13}C] isotopes per indicated molecule. Data representative of two independent experiments. Error bars indicate SEM. Abbreviation: EV, empty vector; LGHL, low glucose high L-lactate; n.s., not significant; MID, mass isotopologue distribution.

Figure S6: L-lactate does not induce apoptosis or NRF-2 signaling. Related to Figure 5 and 6.

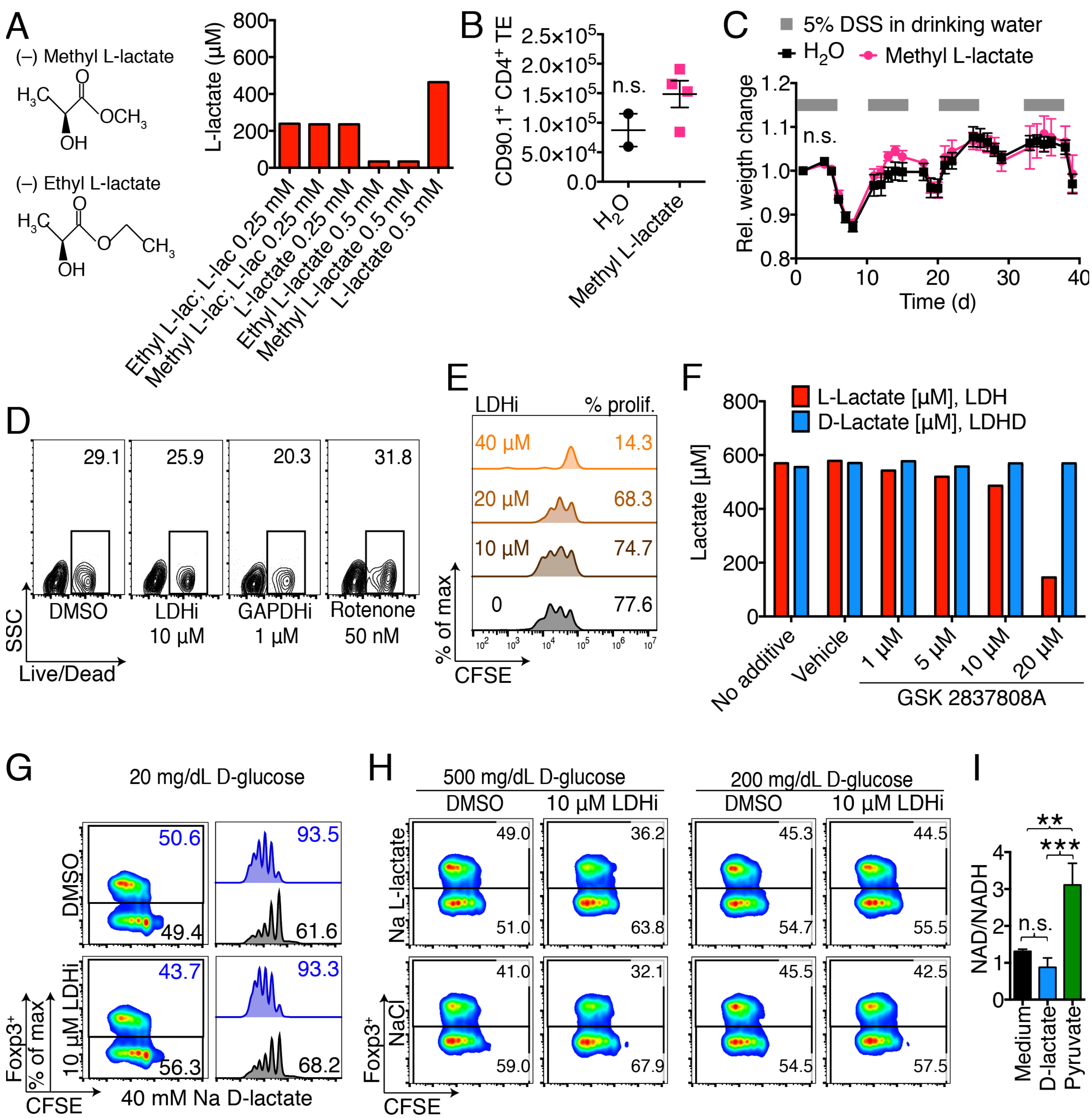


(A) $CD4^{+}CD25^{-}$ Tconv were co-stimulated with CD3ε/CD28 mAb for three days under the indicated conditions, and then underwent apoptosis staining with Annexin V and 7AAD (7-Aminoactinomycin D). Added sodium chloride or sodium L-lactate did not induce apoptosis at up to +40 mM added to the cell culture media. The glyceraldehyde-3-phosphate dehydrogenase inhibitor (GAPDHi) heptelidic acid

(Endo et al., 1985) and HDAC6 inhibitor (HDAC6i) Tubastatin did not cause apoptosis at 1 μM and 0.5 μM, respectively. In contrast, 50 nM rotenone showed mild toxicity. See also supplemental figure S7D for further toxicity data on heptelidic acid & rotenone. (B) $CD4^{+}CD25^{+}$ Tregs, $CD4^{+}CD25^{-}$ Tconv, and $CD90.2^{-}$ antigen presenting cells were isolated from C57BL/6 spleens. The Tconv were CFSE stained, stimulated with anti-CD3ε mAb and irradiated antigen presenting cells, and incubated at the indicated Treg to Teff (effector T cell) ratios for three days. Teff proliferation was assessed by measuring CFSE dilution via flow cytometry. Of note, 50 nM of rotenone do not impair Teff proliferation, but significantly impair regulatory T cell function. In contrast, the GAPDH inhibitor heptelidic acid selectively impairs Teff proliferation, but leaves the suppressive function of Tregs intact. Dimethyl sulfoxide (DMSO) was used as vehicle control. Rotenone data representative of eight, and heptelidic acid data representative of four independent experiments. (C) $CD4^{+}CD25^{-}$ Tconv were stimulated with anti-CD3ε/CD28 beads for 4 d and treated with 3 ng × mL^{-1} TGF-β, 25 U IL-2 × mL^{-1}. Medium control or NaCl was added as indicated. Adding NaCl did not affect iTreg formation. Data pooled from 2-7 independent experiments per group. (D) $CD4^{+}CD25^{-}$ Tconv were stimulated with CD3ε/CD28 mAb coated beads for 4 d ± 20 mM NaCl, Na pyruvate, Na L-lactate, or (–) Ethyl L-lactate, an inert C1 ester of L-lactate (supplemental figure S7A), and subjected OCR measurement by Seahorse. Pre-stimulation in Na L-lactate did increase the oxygen consumption rate (OCR) slightly, but not differently from NaCl control. Data representative of four independent experiments. (E) $CD4^{+}CD25^{-}$ Tconv were stimulated with CD3ε/CD28 mAb coated beads for 4 d, and then subjected to a customized seahorse assay. Adding Na L-lactate (or controls) did not significantly (n.s.) affect OCR. Data representative of two independent experiments. (F) Tconv and Treg were stimulated with CD3ε/CD28 mAb coated beads and 25 U × mL^{-1} IL-2 for 16 h, and mRNA expression of the Nuclear factor (erythroid-derived 2)-like 2 dependent transcription factor NAD(P)H quinone oxidoreductase 1

(*Nqo1*) was determined, and not found to be affected by added 20 mM Na L-lactate or NaCl versus medium control (3/group, one-way ANOVA with Dunnett's multiple comparisons test versus T conv). (G, H): Th17 polarization: $CD8^{-}$ splenocytes were stimulated with CD3ε/CD28 mAb, and cultured with anti-IL-4 & anti-IFN-γ mAbs, TGFβ, and IL-6. Addition of 20 mM Na L-lactate or NaCl increased IL-17 production. Representative (G) and pooled (H) data shown (four independent experiments, * indicates $p<0.05$ using paired one-way ANOVA). (I, J) CD3 and Foxp3 immunoperoxidase slides of BALB/c cardiac allografts from transplant recipients receiving 14 days of 0.2 $mg \times kg^{-1} \times d^{-1}$ rapamycin i.p., and 60 $\mu l \times g^{-1}$ body weight of 150 mM Na L-lactate (9 $\mu mol \times g^{-1} \times d^{-1}$, 10/group) or NaCl (5/group) control were scanned and automatically quantified (Aperio). CD3 (I) and Foxp3 (J) signals were very similar between both NaCl and Na L-lactate injected groups. Error bars indicate SEM.

Figure S7: Methyl L-lactate does not suppress T cell function *in vivo*, and GSK 2837808A does not inhibit LDHD. Related to Figure 6 and 7.



(A): C_1 lactate esters were added to water with or without L-lactate at the indicated concentrations, and L-lactate concentrations measured using recombinant LDH. Neither Methyl L-lactate nor Ethyl L-lactate were metabolized by LDH. (B) B6/Rag1$^{-/-}$ mice were adoptively transferred with 1×10^6 CD90.1$^+$ Tconv and treated i.p. with 60 µl × g^{-1} of 150 mM Methyl L-lactate or H_2O control for seven days. Administration of Methyl L-lactate did not reduce effector T cell (TE, CD90.1$^+$CD4$^+$) homeostatic proliferation (ANOVA with Tukey's multiple comparisons test). (C) C57Bl/6 mice received four 5 d cycles of 5% DSS challenges followed by 5-7 days of recovery. Animals received either the (–) Methyl L-lactate (150 mM) or drinking water control (5/group). Weight was normalized to the weight at the start of the experiment. No significant differences were observed (Student t-test). (D, E) Toxicity evaluation for the LDH inhibitor (LDHi) GSK 2837808A (Billiard et al., 2013; Xie et al., 2014). C57BL/6 CD4$^+$CD25$^-$ Tconv were CFSE labeled, and co-stimulated using soluble anti-CD3ε mAb and irradiated antigen presenting cells for three days, after which T cell proliferation was measured using flow cytometry. (D) LIVE/DEAD® staining identified percentage of dead cells, which was not increased with 10 µM LDHi. Up until 10 µM LDHi, T cell proliferation was unaffected. (E) Up until with 10 µM LDHi, T effector cell proliferation (% prolif.) was not impaired by LDHi. (D, E) Data representative of two independent experiments. (F) L-lactate and D-lactate measurement assays with recombinant LDH and LDHD were repurposed to document LDH specific inhibition by adding 600 µM L- or D-lactate into the respective assay, and evaluating how LDH inhibition reduces the fluorescent NADH readout. Noticeable effects of LDHi on LDH (as expected) were seen by impairment of L-lactate detection at 1 µM and above. D-lactate detection through LDHD was unaffected at the indicated doses. (G) Related for Figure 7C. Na D-lactate was added (40 mM) to low glucose (20 mg/dl) media. C57BL/6 CD4$^+$CD25$^-$ Tconv were CFSE labeled, and polarized to form iTreg (same conditions as in Fig 1A). Na D-lactate impaired proliferation of T cells that did not upregulate Foxp3, but left Foxp3$^+$ iTregs unaffected (NaCl and Na L-lactate conditions please see Figure 6B). In contrast to Na L-lactate, the effect of Na D-lactate on impairing T cell proliferation is less restored by LDH inhibition. (H) High glucose (500 mg/dl) and standard (200 mg/dl) media controls to Figure 6B. In the high glucose conditions, LDH inhibition reduced Foxp3^{+} iTreg formation, while under standard glucose conditions, only minimal effects were observed. (G, H) Data representative of three (G) and five (H) independent experiments. (I) Tconv were isolated from C57BL/6 mice and co-stimulated for 16 h with CD3ε/CD28 mAb coated beads, and 20 mM Na D-lactate or Na Pyruvate, and NAD as well as NADH measured. Pyruvate increased the proportion of oxidized NAD. Error bars indicate SEM.